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Highlights

- An RT-LAMP assay was developed for detection of equine coronavirus
- The RT-LAMP assay was more sensitive than conventional RT-PCR
- Quantitative RT-PCR was more sensitive than RT-LAMP
- RT-LAMP allows for rapid and simple detection of equine coronavirus

Short communication

Rapid detection of equine coronavirus by reverse transcription loop-mediated isothermal amplification

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ABSTRACT

A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for the rapid detection of equine coronavirus (ECoV). This assay was conducted at 60°C for 40 min. Specificity of the RT-LAMP assay was confirmed using several equine intestinal and respiratory pathogens in addition to ECoV. The novel assay failed to cross-react with the other pathogens tested, suggesting it is highly specific for ECoV. Using artificially synthesized ECoV RNA, the 50% detection limit of the RT-LAMP assay was $10^{1.8}$ copies/reaction. This is a 50-fold greater sensitivity than conventional reverse transcription polymerase chain reaction (RT-PCR) assays, but a 4-fold lower sensitivity than quantitative RT-PCR (qRT-PCR) assays. Eighty-two fecal samples collected during ECoV outbreaks were analyzed. ECoV was detected in 59 samples using the RT-LAMP assay, and in 30 and 65 samples using RT-PCR or qRT-PCR assays, respectively. Although the RT-LAMP assay is less sensitive than qRT-PCR techniques, it can be performed without the need for expensive equipment. Thus, the RT-LAMP assay might be suitable for large-scale surveillance and diagnosis of ECoV infection in laboratories with limited resources.

Keywords: Reverse transcription loop-mediated isothermal amplification, Equine coronavirus, RT-PCR, Real-time RT-PCR, Diagnosis

Equine coronavirus (ECoV) has a positive-sense RNA genome and appears to be related to *Betacoronavirus 1* in the *Betacoronavirus* genus of *Coronaviridae* (de Groot et al., 2011). Several ECoV outbreaks were recently reported in the United States (Pusterla et al., 2013) and Japan (Narita et al., 2011; Oue et al., 2011, 2013). Major clinical findings during these outbreaks included fever, anorexia, lethargy, leukopenia and diarrhea. ECoV was detected in patient fecal samples from the United States and Japan, and was also detected in a respiratory sample from Europe (Miszczak et al., 2014). An ECoV experimental challenge study showed that the virus could be detected in fecal samples and nasal swabs (Nemoto et al., 2014).

The current diagnosis of ECoV infection is performed using virus isolation, electron microscopy, serology, and molecular diagnostic methods (Magdesian et al., 2014). Virus isolation, electron microscopy and serological diagnostic methods are not commonly used in clinical laboratories because they are laborious, time consuming, or require specialized equipment. Molecular methods, such as reverse transcription polymerase chain reaction (RT-PCR) assays (Oue et al., 2011) and quantitative real-time RT-PCR (qRT-PCR) assays (Pusterla et al., 2013; Miszczak et al., 2014) have been used to detect ECoV and yield a result within hours. However, these molecular diagnostic tests require expensive specialized equipment, which is a significant barrier to their introduction in

laboratories with limited resources.

Loop-mediated isothermal amplification (LAMP) assays developed by Notomi et al (2000) amplify RNA genomes by reverse transcriptase. Reverse transcription LAMP (RT-LAMP) assays have been widely employed for the detection of several mammalian coronaviruses (Hong et al., 2004; Poon et al., 2004; Chen et al., 2010; Li and Ren, 2011; Pyrc et al., 2011; Ren and Li, 2011; Qiao et al., 2012; Hanaki et al., 2013; Shirato et al., 2014). The RT-LAMP assay can be generally completed within 60 min under isothermal conditions (60–65°C), and results can be analyzed by eye, based on the turbidity or fluorescence of the reaction mixture (Mori et al., 2001; Tomita et al., 2008). The RT-LAMP assay does not require expensive equipment or time-consuming post-reaction work such as gel electrophoresis. These advantages of the RT-LAMP assay might allow its widespread use for the diagnosis of field ECoV infections. In this study, an RT-LAMP assay was developed for the specific detection of ECoV.

Conventional RT-PCR assays were performed using a primer set described previously (ECoV-midf and ECoV-Nr) that targets the nucleocapsid gene and a Qiagen OneStep RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Oue et al., 2011). Thermal cycling involved reverse transcription (50°C for 30 min),

92 then an initial denaturation step (95°C for 15 min), followed by 35 cycles of
93 amplification (94°C for 1 min, 59°C for 1 min, and 72°C for 1 min), and a final
94 extension step (72°C for 10 min). Amplicons were analyzed by agarose gel
95 electrophoresis (FlashGel System for DNA; Lonza Rockland, Rockland, ME, USA) on
96 1.2% (w/v) agarose gels.
97
98 qRT-PCR assays were conducted using a primer set described previously (ECoV-380f,
99 ECoV-522r and ECoV-436p), that targets the nucleocapsid gene, and TaqMan Fast Virus
100 1-Step Master Mix (Life Technologies, Carlsbad, CA, USA) according to the
101 manufacturer's instructions (Pusterla et al., 2013). Thermal cycling involved a reverse
102 transcription step (50°C for 5 min), initial denaturation (95°C for 20 s), and 40 cycles of
103 amplification (94°C for 3 s, and 60°C for 30 s).
104
105 The RT-LAMP primer targeting the ECoV nucleocapsid gene was designed using
106 PrimerExplorer V4 (Table 1; Fujitsu, Tokyo, Japan). The nucleocapsid gene was
107 selected as a target because it is highly conserved among ECoV strains. The
108 nucleocapsid gene of NC99 (accession number: AF251144), Obihiro2004 (AB671298),
109 Tokachi09 (AB555559) and Obihiro12-1 (AB775893) were used to design the
110 RT-LAMP primer used in this study. The reaction mixture was prepared using a

Loopamp RNA amplification kit (Eiken Chemical, Tokyo, Japan) as described previously (Nemoto et al., 2010). Briefly, 2 µl of sample was added to 23 µl of 2× reaction mixture, comprising 12.5 µl of reaction buffer [40 mM Tris-HCl pH 8.8, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% (v/v) Tween 20, 1.6 M betaine and 2.8 mM each dNTP], 0.2 µM each of F3 and B3 primers, 1.6 µM each of FIP and BIP primers, 0.8 µM each of loop F and loop B primers, and 1 µl of enzyme mix (*Bst* DNA polymerase and AMV reverse transcriptase). Reactions were incubated at 60°C for 40 min and then heated at 80°C for 5 min to terminate the reaction. Reactions were evaluated using a real-time turbidimeter (Loopamp LA-320C; Eiken Chemical), and turbidity ≥0.1 was considered positive.

To confirm the specificity of the RT-LAMP assays, amplified products were digested with HpyCH4V (New England Biolabs, Ipswich, MA, USA), a restriction enzyme. Digested products of expected lengths were observed using 2.2% (w/v) agarose gel electrophoresis (FlashGel System for DNA; Lonza Rockland) (Fig. 1). In addition, RT-LAMP assays were performed on samples containing equine viral and bacterial pathogens other than ECoV including equine rotavirus (RVA/Horse-tc/JPN/HO-5/1982/G3P[12], RVA/Horse-tc/JPN/No.1/2010/G3P[12] and RVA/Horse-tc/JPN/No.50/2010/G14P[12]), equine influenza virus

(A/equine/Ibaraki/1/07), equine herpesvirus 1 (01c8, 05c10, and 07c1), equine herpesvirus 2 (08c3), equine herpesvirus 4 (02c21, 04c13 and 06c33), equine adenovirus 1 (05c3), equine rhinitis A virus (NM11), Getah virus (MI-110), *Bacteroides fragilis* (amaero-9), *Clostridium perfringens* (amaero-38), *Clostridium difficile* (amaero-125), *Enterococcus* spp. (Enterococcus spp-1), *Escherichia coli* (Entero-21), *Salmonella* Typhimurium (ST-1), *Streptococcus equi* subsp. *zooepidemicus* (W60 and 122), *Streptococcus equi* subsp. *equi* (CF32 and Hidaka 95/2), and *Rhodococcus equi* (R.equi-6 and ATCC 33701). In addition, 70 nasal swabs were obtained from thoroughbred racehorses (2–6 years old) with fever (≥38.5°C) between January and December 2013. These racehorses were stabled at the Miho Training Center (Ibaraki Prefecture), where an ECoV has yet to be reported. Nasal swabs were suspended in a medium as described previously (Nemoto et al., 2014). Viral RNA and DNA were extracted from nasal swabs, and viruses isolated with a MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany). Bacterial DNA was extracted with an InstaGene Matrix nucleic acid purification kit (Bio-Rad Laboratories, Hercules, CA, USA). The RT-LAMP assay did not amplify any products from the additional viral and bacterial pathogens examined. All 70 nasal swabs collected from thoroughbred racehorses with fever were negative according to the RT-LAMP, RT-PCR and qRT-PCR assays. These results indicate that the RT-LAMP assay does not

149 cross-react with intestinal and intranasal pathogens other than ECoV and therefore, is
150 highly specific for ECoV.
151
152 The analytical sensitivities of the RT-LAMP, RT-PCR, and qRT-PCR assays were
153 evaluated using artificially synthesized ECoV RNA as a positive control. Artificial
154 ECoV RNA was synthesized based on the sequence of the nucleocapsid gene from
155 ECoV strain NC99 as described previously (Nemoto et al., 2014). NC99 was first
156 isolated from a foal with diarrhea in the United States (Guy et al., 2000). Assays were
157 performed twice with quadruplicate samples of artificial ECoV RNA that were serially
158 diluted 10-fold. The detection limits of each assay, where 50% of the diluted samples
159 were positive, were calculated using the Reed and Muench method (1938). The 50%
160 detection limit of the RT-LAMP assay was compared with those for the RT-PCR and
161 qRT-PCR assays using artificial ECoV RNA (Table 2). The 50% detection limits of the
162 RT-LAMP, RT-PCR, and qRT-PCR assays were $10^{1.8}$, $10^{3.5}$ and $10^{1.2}$ copies/reaction,
163 respectively. The RT-LAMP assay was 50-fold more sensitive than the RT-PCR assay;
164 however, the qRT-PCR assay was 4-fold more sensitive than the RT-LAMP assay.
165
166 The RT-LAMP assay was evaluated using fecal samples collected from 82 draft horses
167 (2–9 years old) with anorexia or fever (≥38.5°C) during ECoV outbreaks in 2009 and

2012 (Oue et al., 2011, 2013). These draft horses were stabled at the Obihiro racecourse (Hokkaido Prefecture). Fecal samples were prepared as a 10% suspension as described previously (Nemoto et al., 2014). Viral RNA was extracted from these fecal suspensions using a MagNA Pure LC Total Nucleic Acid Isolation Kit. The RT-LAMP, RT-PCR, and qRT-PCR assays were positive for ECoV in 59, 30 and 65 fecal samples, respectively (Tables 3 and 4). The RT-LAMP assay detected ECoV in 29 additional samples that were negative by RT-PCR assay. In contrast, the qRT-PCR assay detected ECoV in 6 additional samples that were negative by RT-LAMP assay. Results from clinical samples agreed with those when artificial ECoV RNA was used. These results indicated that the RT-LAMP assay was more sensitive than RT-PCR but less sensitive than qRT-PCR.

In this study, the RT-LAMP primers were developed on the basis of currently limited sequence data. Therefore, the RT-LAMP assay may fail to detect variations in ECoV sequences that emerge in the future, indicating RT-LAMP primers must be updated in the future. RT-LAMP reactions can be performed at an isothermal temperature (60°C) within 40 min, and the results can be evaluated easily with the naked eye after adding calcein (Nemoto et al., 2010). Although the RT-LAMP assay is less sensitive than established qRT-PCR assays, its advantage is that it can be completed quickly without specialist equipment. In conclusion, the RT-LAMP assay designed in this study should

187 be suitable for large-scale surveillance and the diagnosis of ECoV infection in
188 laboratories with limited resources.
189

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274

275 **Figure legends**

276 Fig. 1. Restriction enzyme digestion of RT-LAMP products.

277 Undigested RT-LAMP products (Lane 1) and products digested with HpyCH4V (Lane

278 2). M, marker.

279

279

Table 1

Oligonucleotide primers used in this study.

Primer name	Genome binding position [a]	Sequence (5'–3')
F3	29899–29918	GGTACTCCCTCAAGGCTACT
B3	30105–30087	GTGGCATCCTTACCAAGCT
FIP	F1c: 29986–29966	AGAGGCTCTACTGGATGCGCG-
(F1c-F2)	F2: 29923–29940	TGAAGGCTCGGGAAGGTC
BIP	B1c: 30013–30033	TTCCGGCACTAGAACACCCAC-
(B1c-B2)	B2: 30084–30065	GCCAGCACAAGACTAGCAAT
LF	29965–29942	GGAAGTAGATCTGGAATTAGGAAC
LB	30041–30062	GTGACATCTGATATGGCTGATC

[a]Based on ECoV strain NC99 (GenBank Accession number: EF446615)

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Table 2

The 50% detection limits for the RT-LAMP, RT-PCR, and qRT-PCR assays when artificial ECoV RNA was tested.

Assays	RNA copy number (copies/reaction)						50% detection limit
	10^5	10^4	10^3	10^2	10^1	10^0	(copies/reaction)
RT-LAMP	8/8 [a]	8/8	8/8	5/8	0/8	0/8	$10^{1.8}$
RT-PCR	8/8	8/8	0/8	0/8	0/8	No data	$10^{3.5}$
qRT-PCR	8/8	8/8	8/8	8/8	3/8	0/8	$10^{1.2}$

[a]Number of positive samples/number of examined samples

282

283

283

Table 3

Comparison of ECoV detection rates for RT-LAMP and RT-PCR assays using 82 fecal samples.

		RT-LAMP		
		+	–	Total
Conventional	+	30	0	30
RT-PCR	–	29	23	52
	Total	59	23	82

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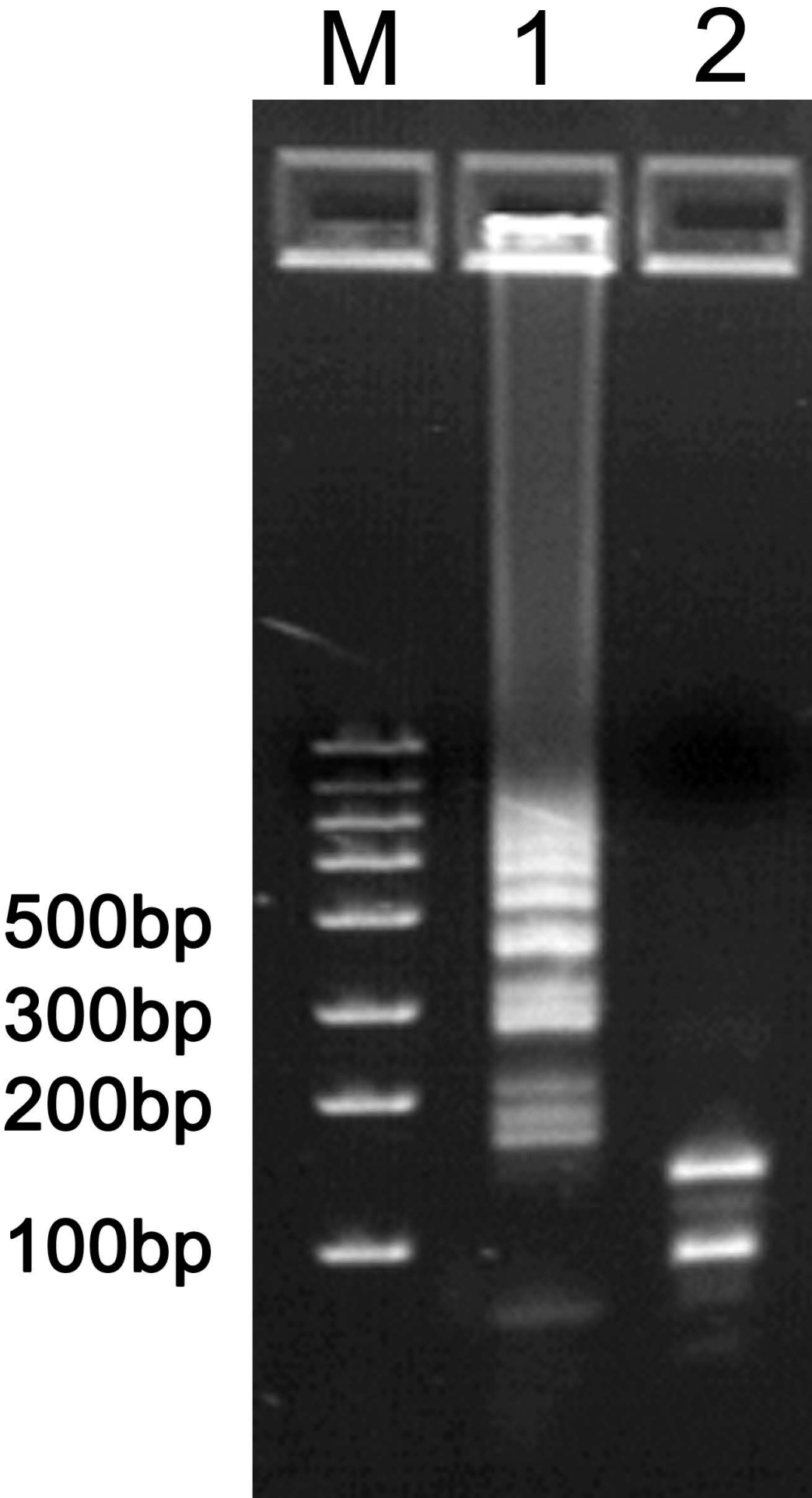
285

Table 4

Comparison of ECoV detection rates for RT-LAMP and qRT-PCR assays using 82 fecal samples.

		RT-LAMP		
		+	–	Total
Real-time qRT-PCR	+	59	6	65
	–	0	17	17
	Total	59	23	82

286


M
1
2
500bp
300bp
200bp
100bp